**Ana Hebe Simões Nunes da Mota**

Licenciada em Engenharia do Ambiente

# The potential of microplastic pellets as a vector to metal contamination in two sympatric marine species

Dissertação para obtenção do Grau de Mestre em
Engenharia do Ambiente

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Co-orientador: Professora Doutora Maria Helena Costa, Professora Associada com Agregação, DCEA, FCT-UNL

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# Agradecimentos

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# Abstract

Microplastics are a global threat to the marine environment due to the ever-growing use of plastic materials, to their abundance and widespread distribution. It is suspected that microplastic size range enables ingestion by many marine organisms. In addition, may release toxicants from paints, coatings and other additives resulting from the manufacturing process, as well as adsorb contaminants, such as metals, and thus act as carry-overs of toxicants. Consequently, there is a concern that microplastics, either pristine or resulting from ageing and fragmentation, can be vectors of marine pollutants via ingestion or release of compounds to the water column, then increasing bioavailability. The present work aimed at assessing the potential of microplastics as vectors of metal contamination to marine organisms by exposing, for 30 days, two sympatric coastal species, the fish *Diplodus sargus* and the decapod crustacean *Palaemon serratus* (juveniles), to pristine and coated polystyrene microplastics (c.a. 1 mm Ø). The coating consisted of a common anti-fouling paint containing copper and zinc. Through a whole-body histopathological screening involving both qualitative and semi-quantitative methods, it was shown that only fish ingested the spherical microplastics, which were present in the intestine, albeit without adverse effects. This indicates that fish are able to cope with microplastic ingestion and passage through the gut. In addition, no effects to metal intoxication were found in either species, suggesting that metal bioavailability was not significantly increased to cause harm. This may result from the fact that either metal is essential, therefore implying high tolerance and reduced toxicity. Altogether, the findings indicate reduced risk of these materials to act as vectors of metals from antifouling paints. Different morphoanatomical aspects and feeding behaviour of both species may explain the observed differences and the selective rejection of microplastics by shrimps.



**Key-words:** Polystyrene microplastics; metal contamination; Fish; Crustacean; Antifouling paints; Copper; Zinc

# Resumo

Os microplásticos são uma ameaça global para o meio marinho devido ao crescente uso de materiais plásticos, à sua abundância e distribuição global. Suspeita-se que o intervalo de tamanho dos microplásticos permita a sua ingestão por parte de muitos organismos marinhos. Além disso, podem libertar compostos tóxicos de tintas, revestimentos e outros aditivos resultantes do processo de produção, bem como adsorver contaminantes, como metais, atuando assim como transportadores de poluentes. Consequentemente, há uma preocupação de que os microplásticos, quer pellets virgens quer fragmentos resultantes do envelhecimento e fragmentação, possam ser vetores de poluentes marinhos por ingestão ou libertação de compostos na coluna de água. Este estudo teve como objetivo avaliar o potencial dos microplásticos como vetores de contaminação de metais em organismos marinhos, expondo, durante 30 dias, duas espécies costeiras simpátricas, o peixe *Diplodus sargus* e o crustáceo *Palaemon serratus* (juvenil), a pellets de poliestireno (cerca de 1 mm de Ø), tanto virgens como revestidos com uma tinta antivegetativa, contendo cobre e zinco. Através de um exame histopatológico de corpo inteiro, envolvendo métodos tanto qualitativos como semi-quantitativos, mostrou-se que apenas os peixes ingeriram os microplásticos esféricos, os quais estavam presentes no intestino, embora sem efeitos adversos. Isto indica que os peixes são capazes de lidar com a ingestão de microplásticos e com a sua passagem pelo intestino. Alem disso, em nenhuma das espécies foram encontrados efeitos de intoxicação por metais, sugerindo que a biodisponibilidade destes metais não foi significativamente alterada para causar danos. Isso pode resultar do fato de ambos os metais serem essenciais, implicando, portanto, alta tolerância e toxicidade reduzida. Globalmente, os resultados indicam um risco reduzido desses materiais para atuar como vetores de metais de tintas anti-vegetativas. Distintos aspetos morfoanatómicos e comportamento alimentar, das duas espécies, poderão explicar as diferenças observadas e a rejeição seletiva por parte dos camarões.

**Palavras-chave:** Microplásticos de poliestireno; contaminação por metais; peixe; crustáceo; tintas antivegetativas; cobre; zinco.

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# List of abbreviations

Cu- copper

DOC- Dissolved organic carbon

H&E- Hematoxylin and Eosin stain

ISO- International Organization for Standardization

MMC- melanomacrophage centre

MSFD-Marine Strategy Framework Directive

NFR- Nuclear Fast Red

PAH. polycyclic aromatic hydrocarbons

PCBs polychlorinated biphenyls

PKD- Proliferative Kidney Disease

R&NFR- Rubeanic acid stain counterstained with Nuclear Fast Red

TC- Tetrachrome stain

UV- Ultraviolet light

Zn-zinc

# 1. Introduction

Marine litter has been a growing and global threat to the marine environment, with plastic debris representing the larger proportion. The durability of plastic that makes it such an attractive material to use also makes it highly resistant to degradation in marine environments (Barnes et al. 2009), this means that when discarded inappropriately, plastic becomes persistent litter, with a long time of degradation, that accumulates in the marine environment. It is estimated that at least 270 000 tons of plastic are currently floating at sea (Eriksen et al. 2014), not including debris ingested by aquatic organisms, or sink in the seafloor.

Even though resistant to degradation plastic materials can be fragmented under marine conditions, mostly due to photochemical degradation, consequent of prolonged exposure to sunlight; and mechanical abrasion (Andrady, 2011; Andrady, 2017; Niaounakis; 2017). The result is the formation of smaller fragments termed microplastics. The denomination of microplastic is attributed to all plastic debris smaller than 5.0 mm, and can be divided into two categories: 1) primary microplastics, that are manufactured at micrometric size, such as pristine ("virgin") plastic pellets, plastics present in facial cleansers and cosmetics (Zitko and Hanlon, 1991), or in industrial abrasives (Gregory, 1996). 2) secondary microplastics, plastic fragments consequent to fragmentation of larger plastic debris. Microplastics represent a significant part of plastics in the marine environment, with an estimate of 51 trillion microplastic debris floating in the sea (Van Sebille et al. 2015), weighting more than 35 500 tons (Eriksen, 2014).

Over the recent years, microplastics have raise concern in the scientific community, because they can probably pose a more serious threat than macroplastic to marine organisms. Microplastic debris usually present the same size-range as natural food items, contributing to their ingestion/filtration from a wide range of marine organism (Barnes et al. 2009). At the same time, microplastics can adsorb and transport waterborne and airborne contaminants such as polycyclic aromatic hydrocarbons (PAHs) polychlorinated biphenyls (PCBs) (Mato et al. 2001; Endo et al. 2005), metals. (Ashton et al. 2010, Holmes et al. 2012; Brennecke et al. 2016) or even pesticides such as DDT (Rios et al. 2007, Frias et al. 2010). In addition, microplastics have the propensity to release additives added to materials to enhance their functionality, like plasticisers, antioxidants, and flame retardants (Barnes et al. 2009, Massos and Turner, 2017). It is estimated that every year 35 to 917 tonnes of chemical additives are released into the marine environment from plastic materials (Suhrhoff et al. 2015).

In this study, the focus is metal contaminated polystyrene microplastic pellets, using antifouling paints as a proxy to metal contamination. Most of the actual antifouling paints use metal compounds as a biocide, especially copper (Voulvoulis, 1999), to prevent the growth of biofouling in immersed surfaces, consequently contributing to a large input of metal compounds in the seawater. In 2009 has been estimated that approximately 3000 tonnes of copper are inputted into sea every year from antifouling paints (Brooks 2009), making this paints one of the major sources of metals release into the marine environment (Almeida et al. 2007), especially in coastal environments where the anthropogenic activities, like marinas and harbours, are frequent.

Metal compounds are known to have the ability of preventing the growth of biofouling but are also contaminants with known significative and harmful effects to aquatic organisms. Even sublethal concentrations of metals such as Zinc and Copper can induce severe damages in several organs, such as in gills (Bayen, 2012; Blewett et al. 2017), hepatic tissue (Heath, 1995; Liu et al. 2011), kidney and spleen (hematopoietic tissues). Metals can also produce defects on sexual development and reproduction (Harris et al. 2001) and can interfere with both neurological and endocrine control systems (Handy, 2003). In addition, metals, are possibly bioaccumulated by marine organisms and can be transferred through trophic levels.

Attending to the reported ability of microplastic pellets to adsorb metallic compounds, it may be inferred that they can act as vectors for the horizontal transport of metals in the marine environment. Metals adsorbed to pellets may yield two pathways of exposure, the microplastics can adsorb the metals from the paint, as reported in the study of Brennecke et al. (2016), and through desorption during digestion, contributing to a dietborne exposure. At the same time, adsorbed metals may be released into the saltwater contributing to an exposure from direct contact between marine organisms and the metal-contaminated water

Considering the highlighted traits, it is possible that metal contaminated microplastic pellets can yield serious threats to marine organisms specially in coastal environments where the anthropogenic activities, may contribute significantly to contamination. Marinas, harbours and industries and urban areas, are common in coastal territories and are significant fonts of both metal and plastic contamination. The coastal environments yield diversified productive and vulnerable ecosystems with a huge ecological and socio-economical relevance, whose deterioration may affect significantly important resources such as fisheries and aquaculture, as well as vital ecological services. Under this perspective, and in order to achieve a “Good Environmental Status”, the Marine Strategy Framework Directive (MSFD) (Directive 2008/56/EC) introduced a series of descriptors, where is established the necessity to monitoring marine contamination, using representative and ecological relevant organisms of the local biota. Fish and invertebrates, due to their complex habitat requirements and their high sensitivity to toxic compounds, are considered relevant biomonitoring organisms for metal pollutants on the integrity of the aquatic environment (Boening, 1999; Chovanec et al. 2003; Costa et al. 2013; Chiarelli and Roccheri, 2014; Authman, 2015). To realistic understand the potential effect, of metal contaminated microplastics in marine environments the same logic as to be present, surveying very distinct, albeit sympatric species, specifically a teleost and a crustacean, enables a wider perspective on potential effects among marine macrofaunal diversity. Accordingly, in this study these organisms are used as a proxy of different macrofauna found in coastal environments

The white seabream *Diplodus sargus* (Linnaeus 1758) is a teleost fish that belongs to the Sparidae family found mostly in coastal rocky reef areas (Planes and Lenfant, 2002) throughout the Mediterranean Sea and along the west African coast to South Africa (Fischer et al. 1987). *D sargus* rarely exceeds 40 cm in size (Macpherson, 1998) and is characterized by a home range with low vertical movement (from shallow water down to >50 m deep), but high-potential horizontal movement (Harmelin, 1987). This species has high commercial value, being one of the most important commercial seabream species in the Mediterranean Sea (Fischer et al. 1987).

*Palaemon serratus* (Pennant, 1777) is a coastal decapod crustacean that inhabits inshore coastal areas, being found in either shallow intertidal pools or deeper subtidal waters (Figueras, 1984). This species has a large distribution, occurring along from North-Western coast of the Atlantic to the Mediterranean Sea (Haig et al. 2014) and is considered a key crustacean species in European coastal marine ecosystems (Silva et al. 2013). In addition to maintaining a very important traditional activity in some fishing communities due to its high commercial value (Haig et al. 2014; Perina et al. 2017), this species is a prey of relevant commercial fish species (e.g. from the family Moronidae and Sparidae) (Madeira et al. 2014), which translate in high economic and social value.

# 2. Objectives

The main objective of this thesis is to evaluate the potential of microplastics as a vector for metal contamination in coastal marine organisms, appraising the histological effects from exposure to metal contaminated pellets in two economic and ecologically relevant sympatric species from distinct trophic groups, the marine teleost, *Diplodus sargus* and the decapod crustacean, *Palaemon serratus*. Microplastics are a major pollutant in the marine environment with the capacity to adsorb and transport metals, and therefore can potentially perform as vectors of metal contamination in marine organisms via the ingestion or the release of compounds to the water column.

This study aims to assess the potential association of metal release from plastic materials, representing realistic and relevant ecological scenarios of contamination, using therefore realistic doses of microplastic diets, an antifouling coating as a proxy of natural contamination, and two ecologically relevant species as representative of the macrofauna of coastal environments.

Specifically, the main objectives of this thesis may be summarized as follows:

- To identify the potential of microplastics as vectors for metal contamination in coastal marine organism;
- To compare potential differential histopathological effects between fish and shrimp;
- To assess effects of microplastic ingestion and associate it with metals released from antifoulants;
- To identify the organs most affected after exposure to coated microplastic pellets;
- To Infer on potential changes of metal bioavailability and their relation to toxicopathic lesions;
- To Infer on potential metal deposition accumulation as a result of exposure to coated microplastic pellets;
- To contribute to histological description of these animals, with emphasis on *Palaemon serratus*.

# 3. Methods and Materials

## 3.1. Organism collection and acclimatization

Individuals from both species were collected from intertidal rocky pools during the low tide at Cabo Raso, West Portugal, in July 2016. Animals were transported to the rearing facilities in plastic containers with a volume of 96 l, with aeration. The animals were acclimatised, for ten days, in glass aquaria, in a ratio of 25 individuals per aquarium. During this time, animals were fed with shrimp crumb every day and with commercial fish food every two days.

## 3.2. Experimental procedure

### 3.2.1 Exposure apparatus

The system consisted of a closed system comprising eighteen glass aquariums, each with the capacity of 25 l. The aquaria were supplied with reconstituted seawater (salinity was maintained at ≈ 34), filtered and re-circulated water (from the sump). The filtering system consisted of a mechanical filter (cleaned daily), connected to a sump equipped with mechanical and biological filtering, followed by a UV filter. The system was kept in an acclimatized room, with a temperature of 20 °C (± 0.5 °C). The constancy of water quality was assured by daily monitoring of parameters.

### 3.2.2 Experimental feed

The experimental diets, were based on shrimp crumb as the main protein source. These diets were formulated after preliminary experiments that compared their suitability with, jelly plus shrimp crumb, jelly plus water from the shrimp and jelly plus commercial fish food. Diets based on shrimp crumbed were determined to be the most suitable in face of better ability to aggregate microplastic spheres with minimal losses to water while retaining, but also which retained attractiveness. For the purpose, the shrimp crumb was crushed in a blender, separated into several portions with the same weight and then frozen. Each portion consisted of 2.19 g of shrimp meal and to each portion were manually blended spheres of virgin polystyrene with a weight of 0.275 mg per particle and 0.7-0.9 mm in diameter (Styropor® P 326, BASF, Ludwigshafen, Germany). A batch of spheres was coated with commercial antifouling red paint (Micron Optima Base YBA953 and Micron Activator YBA953, International Paint Ltd, Southampton, UK). The coating consisted of 25-50% copper oxide and 10-25% zinc oxide. In total, five feeds were formulated: (1) Control diet, containing only shrimp crumb; (2) shrimp crumb plus 8 microplastic spheres, equivalent to 0.1% of the final weight; (3) shrimp crumb plus 16 microplastic spheres, equivalent to 0.2% of the final weight; (4) shrimp crumb plus 8 antifoulant-coated microplastic spheres; (5) shrimp crumb plus 16 antifoulant-coated microplastics spheres. Sphere contents are indicated per portion.

### 3.2.3 Bioassay

Each aquarium was supplied daily with a portion of experimental feed to assure a*d libitum* access to food. Excess feed was aspirated from bottom of aquaria every day. The feeding procedure was supplemented every two days with commercial fish food (supplied *ad libitum*) to avoid nutritional deficiencies. The experimental procedure was conducted through 30 days, after which, random individuals of both species from all experimental treatments, were sampled and prepared to the histological procedure (Table 1). Mortality was recorded throughout the assay and dead or moribund individuals were removed.

Table 1. Summary of individuals from both species collected for histological analyses, per treatment.

| **Treatment diet** | ***Diplodus Sargus*** | ***Palaemon Serratus*** |
|---|---|---|
| [0%] –Blank | 6 | 5 |
| [0.1%] | 7 | 6 |
| [0.1%] * M | 5 | 6 |
| [0.2%] | 6 | 6 |
| [0.2%] * M | 6 | 3 |
| Total | 30 | 27 |

## 3.3. Histological procedure

All fish were fixed in Bouin's solution (10% v/v formalin and 7% v/v acetic acid to which picric acid was added to saturation). Crustaceans were fixed either in Bouin's solution or in Davidson's fixative (10% v/v formalin, 10% acetic acid, 30% v/v ethanol). Whole-animals were immersed in the fixative solution for 36 hours at room temperature, followed by washing in tap water (24 h) to remove the excess fixative and archiving in 70% ethanol.

In order to preserve microplastic integrity, since these are easily degraded by standard xylene-based protocols, samples were processed according to the protocol of Gonçalves et al. (2017). In brief, fixated organisms were dehydrated with two baths of isopropanol 95% for 15 min, followed by three baths of 100% isopropanol (1 h each) as final dehydrating agent and intermediate infiltrator. These three final baths were performed under low-pressure vacuum conditions to enhance infiltration. The samples were then embedded in molten paraffin, for at least 24 h. After 24 h, the paraffin was replaced to remove all traces of isopropanol, with the assistance of low-pressure vacuum as well,

using a desiccator and a pre-heated water bath arrangement in which the glass vials holding the samples were placed.

The final paraffin-embedded samples were cut in sections with a thickness of 5 µm, using a Jung RM2035 model rotary microtome (Leica Microsystems). At least three slides per individual were obtained, each allocating 3-4 serial whole-body longitudinal sections along the median axis of the animal. The sections were mounted using a water bath (set between 35° and 40°) on regular glass slides employing a standard albumin + glycerol aqueous adhesive.

The slides were deparaffinated with several baths of isopropanol alternated with heat to promote melting of paraffin, as isopropanol is less efficient than xylenes. The slides were then rehydrated in a regressive series of isopropanol (100%, 95%, 70%), and brought to water through a final immersion in Milli-Q grade water (16.2 MΩ.cm). The deparaffinated samples were stained with Haematoxylin (Harris') and counterstained with alcoholic Eosin Y (H&E) for general structural analyses. Rubeanic acid and counterstained with Nuclear Fast Red (NFR) for metal especially Cu. Tetrachrome solution, first stained with Alcian Blue, followed by Weigert's Iron Hematoxylin dye differentiated with a ferric chloride solution, and counterstained with van Gieson's dye, for histochemical analyses. See Costa (2018), for procedural details.

### 3.4. Preliminary histopathological observations

The analyses were carried out using a DMLB model microscope coupled to a DFC480 model digital camera (all from Leica Microsystems, Germany). A preliminary qualitative histopathological analysis was performed on the internal structure of the animals to look for general histopathological alterations and clear signals of microplastic ingestion. This qualitative scrutiny was used to select target organs and define the list of histopathological traits to be considered for the semi-quantitative approach.

The target organs for the histopathological analysis of both species were selected attending to their biological importance and their known interaction with toxicants, especially metals. In fish, the selected organs were the hepatopancreas, kidney, gills, spleen, and the organs from the digestive tract. In shrimps the organs were the ones comprising the digestive tract, the gills and the green gland.

### 3.5. Histopathological condition indices

Individual semi-quantitative histopathological condition indices *($I_h$)* were calculated for each individual, accordingly to the methodology proposed by Bernet et al. (1999) and modified by Costa et al. (2013). The individual histopathological condition was estimated as the product between the weight (w) of each histopathological alteration, which is based on its biological significance, and the score value (a), which is a numerical attribute that reflects the degree of dissemination of the alteration within the surveyed organ. The weight of each pathological alteration ranged between:

1- the lesion is reversible, has low severity; 2 - the lesion is reversible in most cases if the stressor is neutralized; and 3 - the lesion is generally irreversible, leading to partial or total loss of the organ/cell function (Bernet et al. 1999) and were attributed accordingly to Costa et al. (2013). The score ranged between 0 (unaltered condition or unobserved lesion); 2 (infrequent occurrence); 4 (moderate occurrence); or 6 (severely disseminated/diffuse). The individual histopathological condition indices were estimated accordingly to the formula proposed by Costa et al. (2013):

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j} \qquad \text{[Equation 1]}$$

Where $I_h$ is the histopathological condition indice for the individual *h;* $W_j$ the weight of the histopathological alteration *j;* $a_{jh}$ score attributed to the individual *h* for the alteration *j* and $M_j$ is the maximum attributable value for the alteration j, i.e., weight × maximum score. The equation's denominator normalizes $I_h$ to a value between 0 and 1, thus permitting comparisons between distinct situations such as different organs, species or experimental set-ups (Costa et al. 2013). A series of blind reviews was performed for quality assessment (AQ) in at least 25% of the slides to validate the findings. The results from the blind review differed from the preceding analyses by < 12%.

### 3.6. Statistical analyses

After the invalidation of the parametric presupposes in the statistical data, determined through the Leven's test to homoscedasticity (homogeneity of variances) and the Kolmogoroff-Smirnoff's to ascertain normality of data, non-parametric tests were used. The Kruskal Wallis ANOVA-by-ranks and Mann-Whitney's *U test* were used for multiple and pairwise comparisons between treatments. Wilcoxon's matched-pair test was employed to compare between organs. Spearman's rank-order correlation (Spearman's R) was used to test correlations between biometric data and histopathological condition indices. The significance level was set at 0.05 for all analyses. Statistics were performed with IBM SPSS Statistics for Windows, Version 22.0, following Zar (1999).

# 4. Results

Overall mortality during the bioassay was different between species. Fish presented some mortality and moribund individuals (data not shown) for unknown causes, while shrimps had no mortality at all. The length of the surveyed fish ranged between 2.7 and 4.8 cm and the weight from 0.35 to 1.87 g.

## 4.1. Microplastic ingestion

While fish showed clear proof of microplastic ingestion (in both animals treated with pristine and metal-coated pellets) and possible accumulation in the gut, as displayed in Fig. 1, *P. serratus* did not reveal any signs of microplastic uptake.

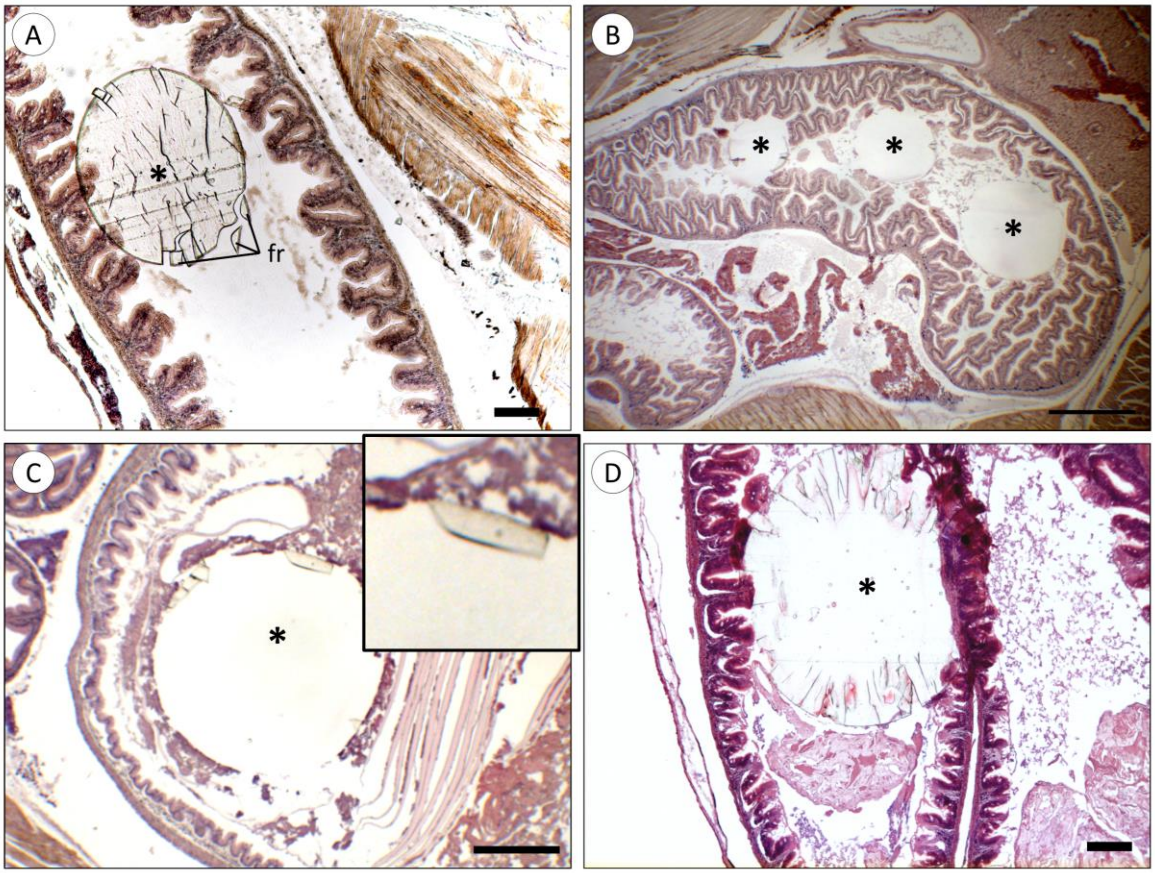



Figure 1. Plastic ingestion by *D. sargus*: A) Plastic pellet (*) in the posterior part of the intestine with plastic fragments from sectioning (fr) (scalebar=200µm, TC). B) pellet (*) accumulation in the anterior part of the intestine (scalebar=500µm, H&E). C) Evidence for plastic pellet (*) in the stomach. Most of the material was damaged during sectioning but the outline and debris are clearly visible (scalebar=200µm, H&E). Inset: Fragment of microplastic pellet still in its place. D) A microplastic pellet (*) compressed between the intestinal wall (scalebar =100µm, H&E).

### 4.2. Copper histochemistry

Metal deposits were found in all samples from both species, even in the blank(b). However, their number and size were variable among the groups, without obvious differences between organisms exposed to metals and their respective controls. In fish, metallic deposits were often found in the intestine, gills, and hepatopancreas (Fig 2). In the intestine, most of the deposits were placed inside goblet cells or in the connective tissue of the submucosa. In gills, deposits were frequent in the epithelial cells of the lamellas, and in the absorptive tissue of the opercular cavity especially in the goblet cells, as demonstrated in Fig 2.C and D. The metal deposits found in the hepatopancreas were dispersed within the hepatic parenchyma.

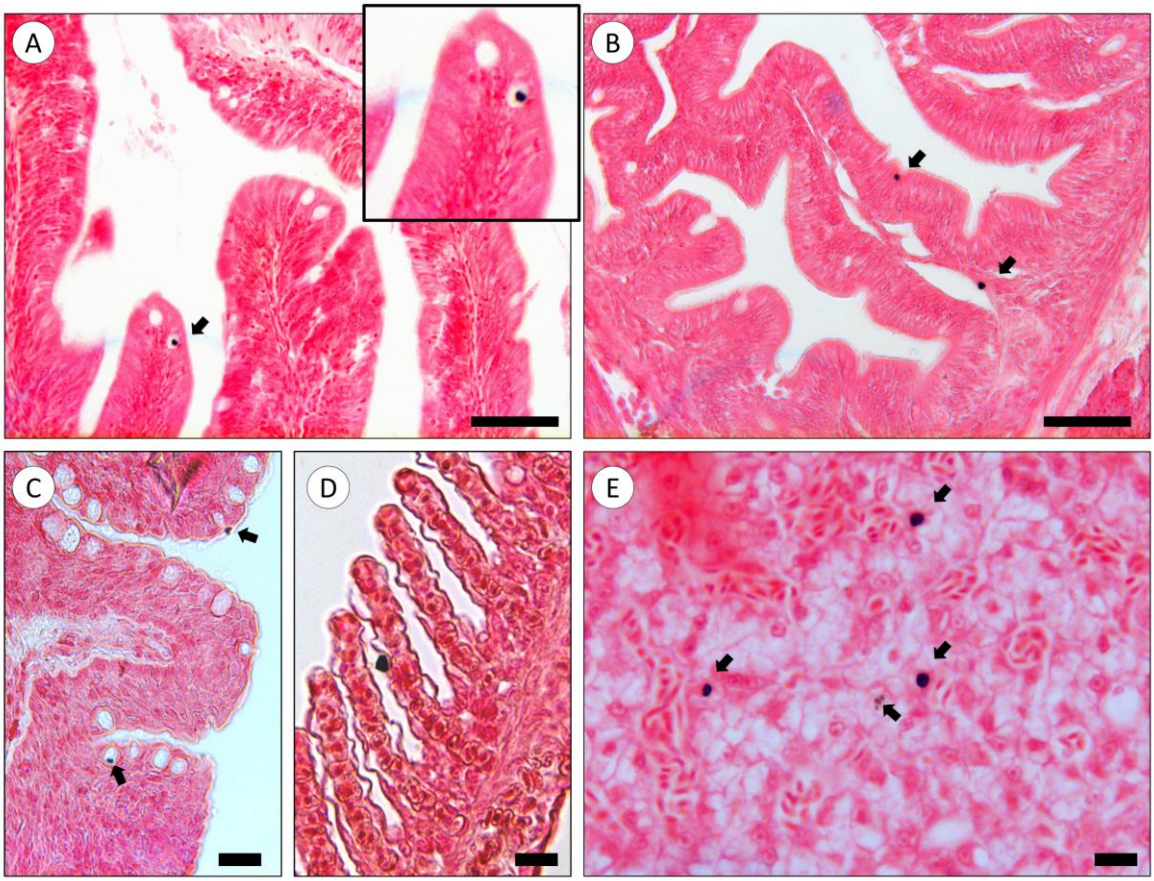



Figure 2. Metal deposits in the surveyed fish (R&NFR): A) Metal deposit within a goblet cell of the intestine villi (arrow) (scale bar= 50µm) Inset: Details of metal deposit. B) Metal deposits in the intestine villi note the different location of the deposits both in the epithelial cells (ep), and in the connective tissue (ct) (scale bar= 50µm). C) Metal deposits in the opercular cavity (arrow) (scale bar= 10µm). D) Metal deposit in gill lamellae (scale bar = 10µm). E) Metal deposits in the hepatic parenchyma (arrows) (scalebar=10 µm).

In shrimp, metal deposits were found in the hepatopancreas, stomach, gills and in the green gland. Within the hepatopancreas, the deposits were present, in B-cells and R-cells, as displayed in Fig.3. Gills presented metal deposits in the lamellar epithelial cells. In the stomach, the deposits were frequent within the cardiac chamber, specifically in the epithelial cells. In the green gland deposits were rare and when present they presented small size.

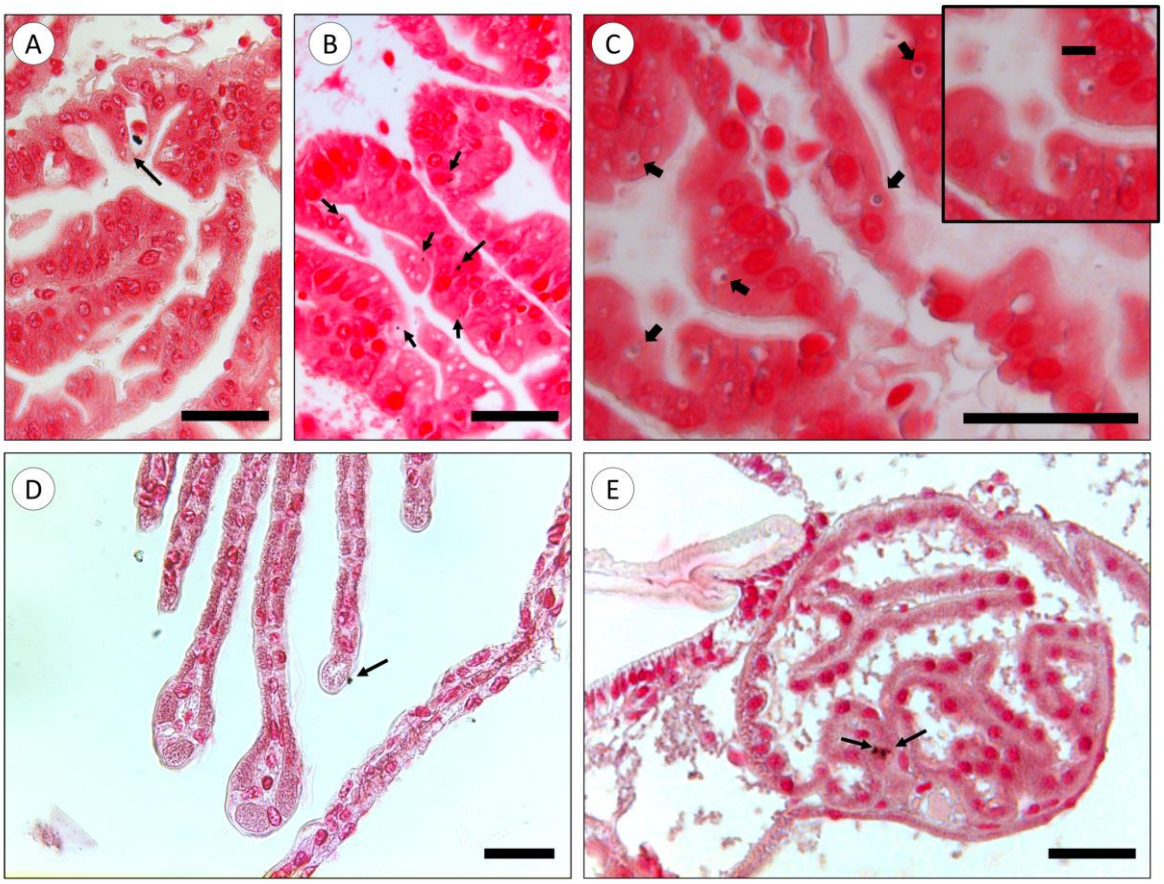



Figure 3. Metal deposits in shrimps (R&NFR): A) Metal deposits in B-cell of a digestive tubule of the hepatopancreas (arrow) (scalebar= 50µm). B) Metal deposits dispersed in the hepatopancreas (arrows) (scalebar=50 µm). C) Spherules of metal in the vacuoles of R-cells (arrows) (scalebar=50µm); Inset: Detail of spherules (scalebar=10µm). D) Metal deposit in the epithelial cells of the lamellas (arrow) (bar=10µm). E) Metal deposits in the green gland (arrows) (bar=50µm).

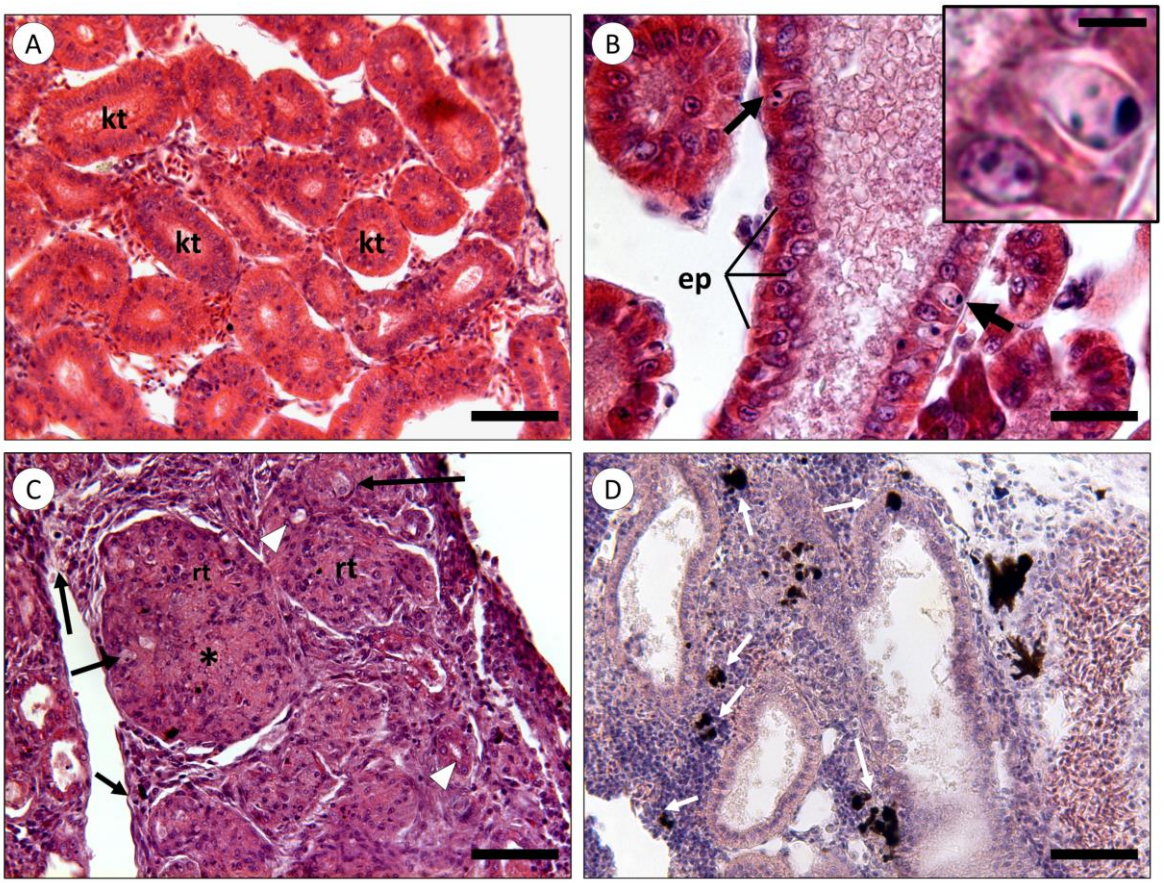
### 4.3. Qualitative analysis

The selected organs thru preliminary qualitative scrutiny to whole-body individuals, were examined to identify the prevalence of any external infection that could perform as a confounding factor and at the same time to identify the most relevant biomarker to include in the semi-quantitative analysis.

In fish, of the selected organs, displayed in Fig.4, only the trunk-kidney showed clear signs of infection and consequent unnormal histological condition. Were identified significant and relevant biomarkers in all organs and categorized into three reaction patterns.


Figure 4. Whole-body longitudinal section of *D. sargus* highlighting internal structure: Opercular cavity (oc), Gills (g), Oesophagus (O), Hepatopancreas (h), Stomach (S), Intestine (I), Kidney (K) (scalebar=500µm, TC).

Most samples, shown clear signs of Proliferating Kidney Disease (PKD). This is a compromising and common disease that affects both kidney tubules and the surrounding hematopoietic tissue. This disease is caused by a myxozoan parasite, in this particular case, possibly by *Leptotheca sparidium n. sp.* ( Sitjà-Bobadilla, and Alvarez-Pellitero, 2001), Fig.5. This organism enters in the host via gills and later proliferates in the kidneys, inducing an intense defence reaction of the host (see Hedrick et al. 1986; Scholz, 1999; Alvarez-Pellitero, 2004; Tops et al. 2005 for a review), leading to the occurrence of histopathological traits, like, the proliferation of defence cells (melanomachophages) or significant regressive and progressive alterations. As the inclusion of this organ in a quantitative analysis could result bias the results, it was excluded from the semi-quantitative analysis.



◀ Figure 5 Trunk kidney of the surveyed fish: A) Normal histology of fish kidney showing regular kidney tubules (Kt),(scale bar=50 µm; TC). B) Tubule infected with PKD, note the inclusions of *myxozoa parasite* in the epithelial cells (ep) of the tubule (arrow) (scale bar=50 µm, TC). Inset: detail of *myxozoa* inclusion (bar=10µm). C) Regressing tubules (rt) formed by cloudy-swollen and necrotic cells, with lumen reduction (arrowhead) and disappearance (*), note the infiltration of defence cells (arrows) (mostly macrophages) in both kidney tubules and hemopoietic tissue. (scale bar=50µm, TC). D) MMC centres (arrow), resulting from an intense defence reaction to the parasite (scale bar=50µm, H&E).

In shrimps, none of the selected organs presented any signs of infection. In fact, in this analysis no significant histopathological alterations could be found in none of the surveyed organs. In gills, no differences were detected between control (C) shrimps, and the ones exposed to metal (M), both in lamellas or in the haemocoelic space, as visible in Fig. 6

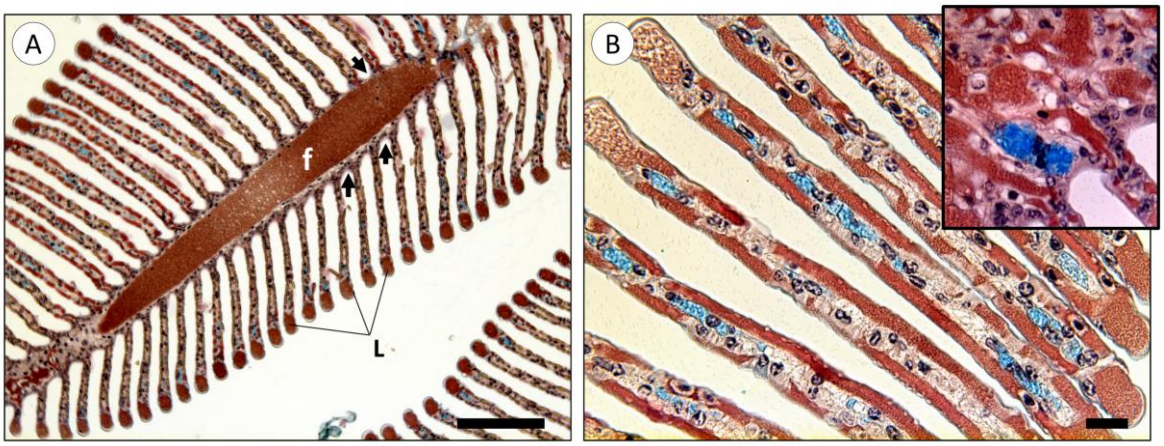



Figure 6 Gill of *P. serratus* exposed to metal coated pellets. A) structure of the gills, with defined filament (f) and the attached lamellas (l) (bar=50µm). B) Detail of the lamellas (bar=10µm) Inset: Haemocelyc space.

The general structure of the fore and midgut of shrimp digestive tract are displayed in fig.7, a longitudinal section of *P. serratus*. In similarity to gills no differences were found between the digestive tract of organisms exposed to different diets, again without the presence of significant histopathological traits in any of the surveyed organs. Considering the lack of enough biomarkers to enable a comparison between exposed and non-exposed shrimps, this species was excluded from the semi-quantitative analyses. Still a histological description of *P. serratus* digestive tract is provided in this section, since it seems to exist a lack of information in literature about the internal structure of this species.

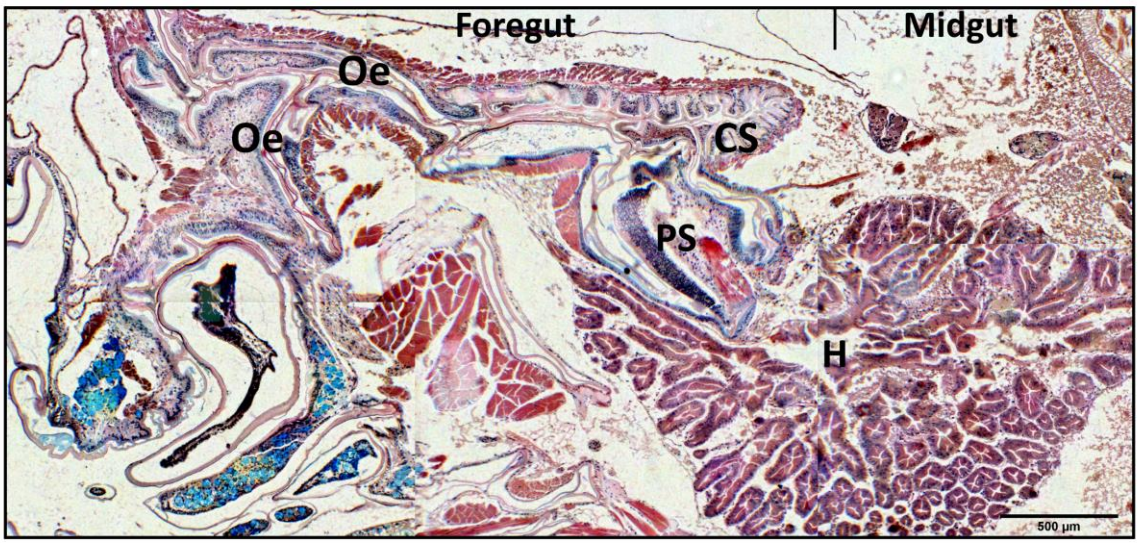


Figure 7 Digestive tract of *P. serratus*: Foregut: Oesophagus (oe) Cardiac Stomach (CS), Pyloric Stomach (PS); Midgut: Hepatopancreas (h), (scalebar=500 µm, TC).

As expected to decapod crustaceans, the entrance of the oesophagus presented extreme lubrication by a mucus rich in acid mucopolysaccharides which accordingly to Vogt, (1996) are secreted by subtegumental glands (Fig. 8). The oesophagus lumen displayed a coat of cylindrical basal epithelium cells, with clear cytoplasm. Parallel to other crustaceans (Ceccaldi, 1989; Vogt, 1996, Sousa, 2006), this epithelium was recovered by a thin hyaline article composed primarily of chitin. Even though most of the samples, showed a moderate epithelium lifting in the oesophagus, this trait has been established as a fixation artefact and not as histopathological lesion. The oesophagus ended in a connection with the first chamber of the stomach, the cardiac part. This chamber presented a sac-like structure, and similar basal epithelium cells to the oesophagus, with a chitin layer or their apical surface. However, in the cardiac stomach this layer presented a serrated form as displayed in Fig 8D. The epithelium was underlain by a layer of dense connective tissue and surrounded by circular and longitudinal striated fibres. The serrated and rigid chitin structure, and the developed muscle helps to grind the digesta, upon the peristaltic movements. The cardiac stomach presented a separation from the second chamber of the stomach, the pyloric one, by an extremely muscled valve, called cardiopyloric valve. This valve regulates the movement of digesta between the cardiac and pyloric chambers. In the pylorus part, was evidenced a complex of gland filters. Contrary to the oesophagus or the cardiac stomach, in the pyloric stomach, the chitin layer covering the apical surface of epithelial cells was absent.

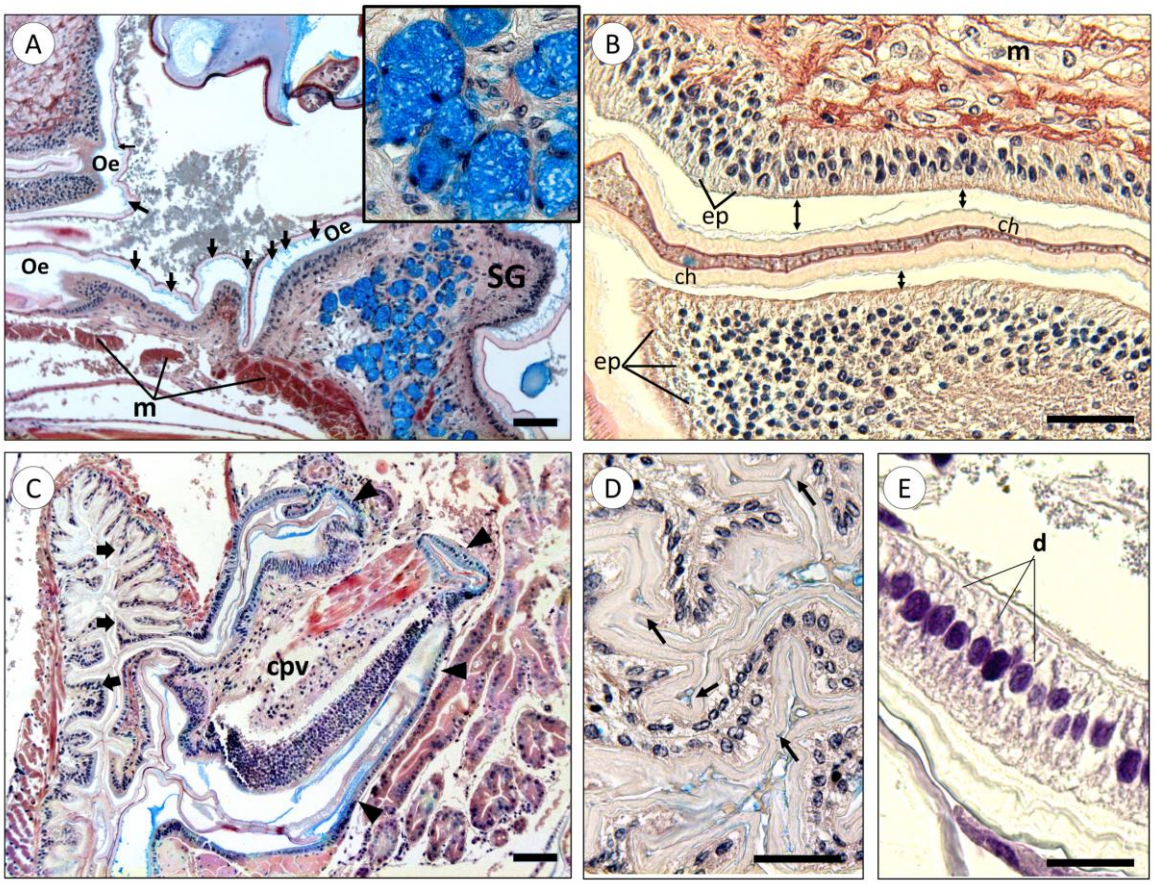



Figure 8. Digestive tract of *P.serratus*: A) general structure of oesophagus with subtegumental glands (SG) and muscle (m), note the acid mucos (arrows) lubricating the oesophagus (Oe) (scalebar=200µm, TC), Inset: acid mucopolysaccharides (severely stained with alcian blue). B) histological structure of oesophagus: note the chitin (ch) layer on the apical surface of the epithelial cells (ep) with epithelium lifting (double arrow) and muscles (m) (scalebar=50µm, TC). C) General structure of the stomach with folds of epithelial tissue in the cardiac chamber (arrows) the cardiopyloric valve (cpv) and the pyloric filter (arrow head) (scalebar=200µm, TC). D) Details of cardiac stomach, note the chitin layer recovering the epithelial cells (ep) presenting a serrated form (arrows) (scalebar=50µm, TC). E) D) Detail of the filter of the pyloric stomach, constituted by digestive cells (d) and lined by a basal membrane (bm) (scalebar=50µm, H&E).

In the midgut, the only analysed organ was hepatopancreas. In this gland, each digestive tubule presented a variable number of different epithelial cell types, like E-cell also called embryonic cells, undifferentiated cells involved in mitotic activity (Franceschini-Vicentini et al. 2009), R-cell associated to the storage of glycogen and lipids, F-cell (fibrillar) and B-cell responsible for intracellular digestion (Al-Mohanna & Nott, 1986). There were no obvious differences in cell numbers or morphology in the digestive tubules between the individuals of different diet groups. In general, epithelial cells were not consistent in shape or size, no matter of the diet type.

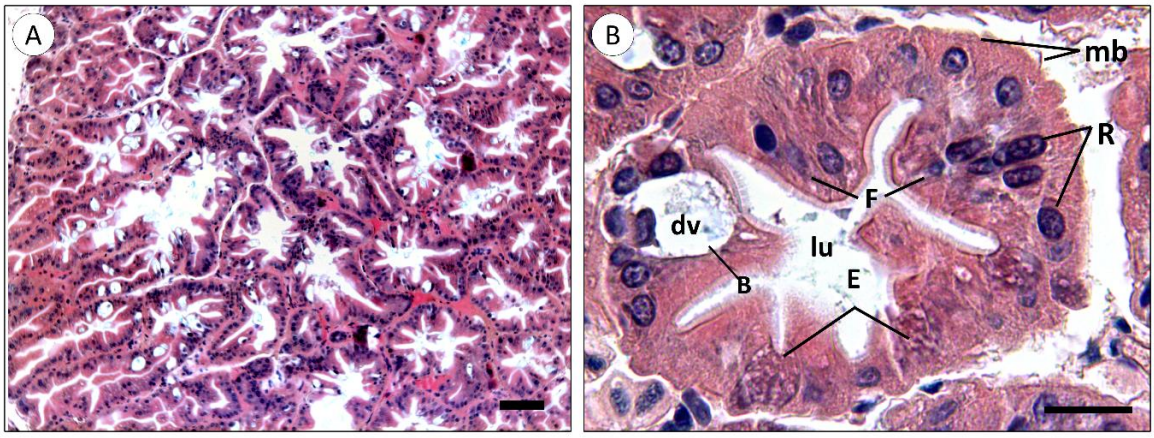



Figure 9 Hepatopancreas of *P. serratus*: A) General structure of hepatopancreas, note the variable size of the digestive tubules (scalebar=50 µm, TC). B) Detail of a digestive tubule of hepatopancreas constituted by E-cells- (E), B-cell (B) with a large digestive vacuole(dv), F-cells (F) and R-cells (R), note the digestive tubule is surrounded by a basal membrane (mb) (scalebar=25 µm, TC).

### 4.4. Semi-quantitative histopathological analysis in *D. sargus*

The respective histopathological alterations were classified into three reaction patterns: R1- inflammatory response-related alterations, which covers both circulatory disturbances and inflammatory responses, R2- regressive and R3- progressive alterations.

#### 4.4.1 Hepatopancreas:

Considering this organ is constituted by two tissues with different structure and function, the analysis of each parenchyma was made separately. The normal hepatic structure was characterized by a rosette arrangement of well-defined hepatocytes, disposed in a simple layer around a sinusoid (hepatic capillaries), Fig.10B. The hepatocytes, revealed a polyhedric shape, regular cell size and a translucent cytoplasm with few or absent inclusions. The normal pancreatic tissue was characterized by the presence of acinar cells with large spherical nuclei, dark basophilic cytoplasm with of brownish/eosinophilic zymogen granules, Fig.10C. The weights of the estimation of indices for the hepatic and pancreatic parenchyma are given in Table 2 and in Table 3 respectively.

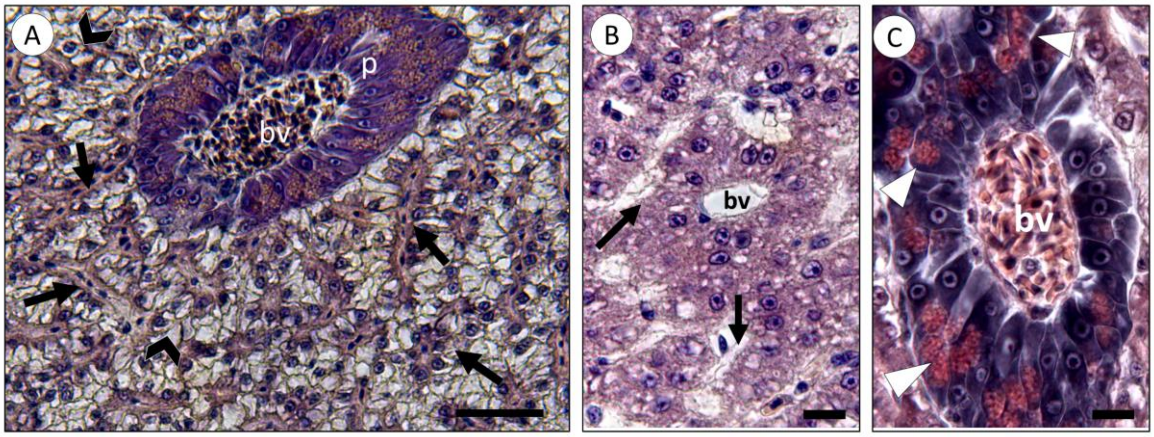



Figure 10 Hepatopancreas of *D. sargus* from the blank treatment. A) normal structure of hepatic and pancreatic parenchymas note the hepatic tissue with well-defined hepatocytes (arrowhead) and sinusoids (arrows) and the ▶

◀ pancreatic tissue (p) around a blood vessel (bv) (scalebar=50µm, H&E). B) Detail of the Hepatic parenchyma: note the hepatocytes disposed around sinusoids (arrows) and a larger blood vessel (bv), probably an arteriole (scalebar=10µm, H&E). C) Pancreatic parenchyma: note the pancreatic tissue around a blood vessel (bv), acinar cells (ac) and eosinophilic zymogen granules (arrowheads) (scalebar=10µm, TC).

Table 2 Histopathological alterations observed in the hepatic parenchyma of *D. sargus* and their respective condition weight (w)

| Reaction Pattern | Histopathological alteration | Weight |
|---|---|---|
| 1.Circulatory disturbances/ Inflammatory responses | Hyperaemia | 1 |
| | Haemorrhage | 1 |
| | Leukocyte infiltration | 1 |
| 3-Progressive alterations | Hyaline degeneration | 2 |
| | Fat degeneration | 1 |

In the hepatic tissue were found alterations from the first (R1) and third (R3) reaction pattern as displayed in Fig.11. Eosinophilic inclusions were the most significant progressive alteration found since they were present in most of the individuals of all treatments. Still, no obvious differences were detected between groups, both in the degree of dissemination or in the number of occurrences. The altered hepatocytes showed a tendency to retain eosin in the cytoplasm and to lose their typical polyhedric shape (Fig.11 C/D). These inclusions delimitated by a membrane and ellipsoidal in shape were present in small numbers inside the cells. The presence of eosinophilic bodies termed hyaline degeneration is commonly associated with strongly damaged tissue (Costa et al. 2009). Hepatocyte lepidic vacuolation was another common progressive hepatocellular alteration, present in 88% of the samples. This vacuolation was variable, in the vacuoles sizes and in its degree of diffusion within the hepatic tissue, without a clear trend regardless of the treatment type, or concentration. Within inflammatory response-related alterations, the most common trait was hyperaemia, evidenced by sinusoidal swelling, and blood congestion. This trait was most of the time associated with the presence of hyaline degeneration or fat vacuolation (fig.11B) and was present in most samples of all treatments. Haemorrhage was restricted to small foci, typically around blood vessels and was found in a limited number of samples (12%). Infiltration of defence cells was also present in a reasonable number of samples (64%), however, was limited to small foci within the hepatic parenchyma, normally associated with cell dead (necrosis or apoptosis) as displayed in Fig.11A.

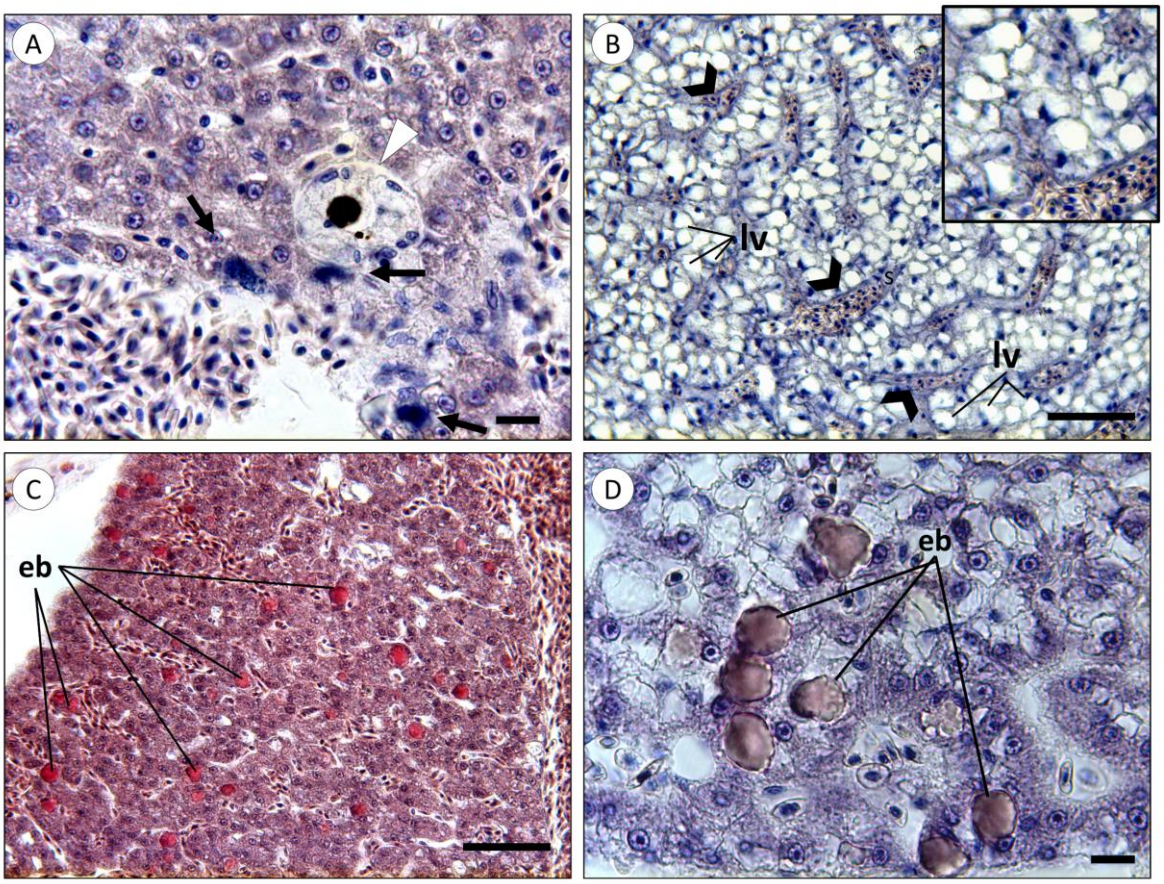



Figure 11 Histopathology of the hepatic parenchyma of *D. sargus:* A) Focal dead cell (necrosis or apoptosis) (arrowhead) with infiltration of defence cells (arrows) (scalebar=10µm, H&E). B) Hepatocyte lipidic vacuolation (lv), note the loss of shape of the hepatocytes with the nuclei compressed through the membrane, because of the vacuoles size and the moderated hyperaemia, evidenced by sinusoid swelling (arrowhead) (scalebar=50µm, H&E). Inset: Detail of lepidic vacuoles and hyperaemia. C) Moderate hyaline degeneration, with inclusions of eosinophilic bodies (eb) dispersed all over the hepatic parenchyma (scalebar=50µm, TC). D) Detail of eosinophilic inclusions (eb) note their ellipsoidal shape and the enclosing membrane (scalebar=10µm, H&E).

In the pancreatic tissue, infiltration of defence cells was the most frequent surveyed trait. Small foci of leukocytes and other defence cells were observed in the majority of the samples, however, in 16% of them, the extension of this foci was more significant. Atrophy of the acinar cells was observed in almost 45% of the samples. As displayed in Fig. 12, in atrophied cells the zymogen granules were absent, with the loss of cellular structure. Foci of cell death (necrosis or apoptosis) were present in the pancreatic tissue of 12% of the samples, evidenced by pyknotic nuclei (Fig12B).

Table 3 Histopathological alterations observed in the pancreatic parenchyma of *D. sargus* and their respective condition weight (w)

| Reaction Pattern | Histopathological alteration | Weight |
|---|---|---|
| 1.Circulatory disturbances/ Inflammatory responses | Infiltration of defence cells | 1 |
| 3- Progressive alterations | Atrophy | 2 |
| | Necrosis | 3 |

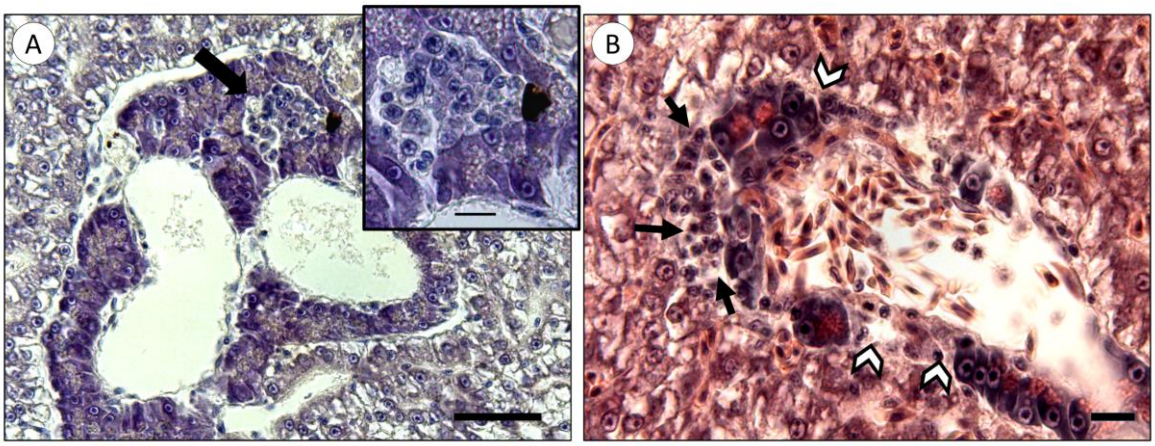



Figure 12 Histopathology of the pancreatic parenchyma of *D. sargus*: A) Infiltration of defence cells, with inclusions of defence cell (arrow) like leukocytes and MMC (scalebar=50µm, H&E). Inset: Detail of infiltration of defence cells (scalebar=10 µm). B) Details of progressive alterations within the pancreatic tissue, note the acinar cells necrosis evidenced by the pyknotic nuclei (arrow) in the necrotic zone, and the lack of zymogen granules in the atrophied (arrowhead) acinar cells (scalebar=10µm, TC).

### 4.4.2 Gills

Most of the surveyed gills showed a normal architecture with well-defined lamellae attached to a filaments and presence of goblet and chloride cells in the interlamellar space, Fig.13. The histopathological screening revealed only few significant lesions and alterations regardless of the treatment type. The weights of the estimation of indices are given in Table 4.

Table 4 Histopathological alterations observed in the gills of *D. sargus* and their respective condition weight (w)

| Reaction Pattern | Histopathological alteration | Weight |
|---|---|---|
| 1.Circulatory disturbances/ Inflammatory responses | Oedema | 1 |
| | Hyperaemia | 1 |
| | Aneurysm | 2 |
| | Leukocyte infiltration | 1 |
| 2- Regressive alterations | Epithelial cell hypertrophy | 2 |
| | Lamellar fusion | 2 |
| 3- Progressive alterations | Interlamellar space hyperplasia | 2 |

Inflammatory response-related alterations were the most frequently traits found in this organ. Within these alterations, the most significant was epithelium lifting, caused by the formation of oedemas, through liquid accumulation This trait was found in all samples from all treatments including the blanks (B) and its degree of dissemination was variable, however slightly more pronounced in fish exposed to metals (0.2 coated pellets). Hyperaemia was another frequent trait, evidenced by blood congestion causing lamellar swelling. This trait was present in a variable number

of lamellas with a low severity and a variable degree of dissemination, throughout the organ. The infiltration of leukocytes and other defence cells, like eosinophilic (basic) granulocytes, was present in most samples but expressed mainly in small foci in the interlamellar space, or in epithelial cells. Some regressive alterations, like lamellar fusions or hypertrophy of the epithelial cells, were also found but were always limited to reduced number of lamellae. Focal lamellar fusion was present in a relatively reduced number of samples (10%). This trait was characterized by the loss of normal architecture of the lamellae, resulting in fusion with the adjacent lamellae. Focal hypertrophy of the epithelial cells was found in 12% of the samples and was characterized by an abnormal size of the lamellar epithelium, as displayed in Fig.13E. Regarding progressive alterations, only hyperplasia of epithelial cells was found in the interlamellar space, evidenced by an abnormal proliferation of non-differenced or immature cells. This trait was present in 8% of samples and was restricted to some lamellas within a filament.

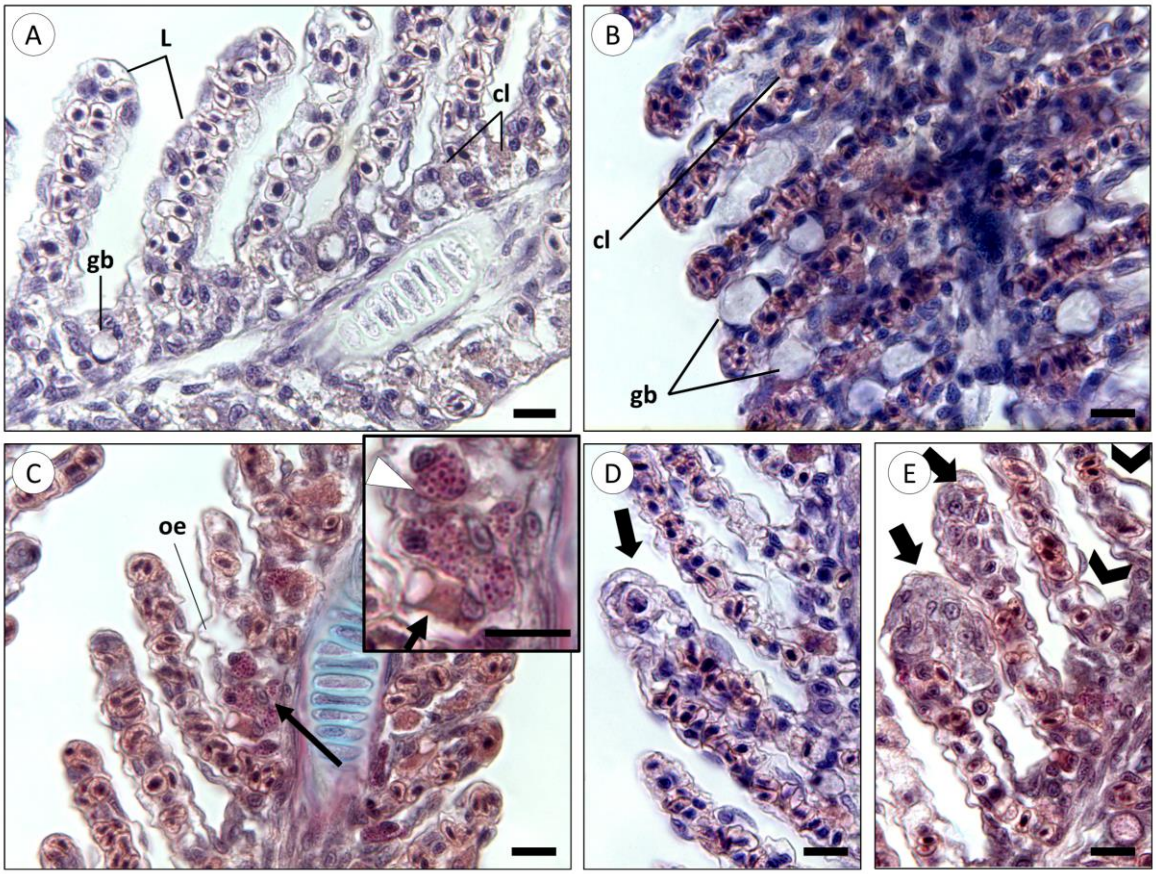



Figure 13 Histopathology of the gills of *D. sargus*: A) Gills normal condition with well-differentiated lamellae (L) with chloride (cl) and goblet cells (gb) in the interlamellar space (scalebar=10µm, H&E). B) Hyperplasia of the interlamellar space, note the proliferation immature cells, like goblet (gb), and chloride (cl) cells occupying the lamellar space (scalebar=10µm, H&E). C) Infiltration of defence cells note the formed oedema (oe) through infiltration of granulocytes (arrow) (scalebar=10µm, TC). Inset: Detail of the granulocytes (scalebar=10 µm). D) Lamellar fusion with the fusion of the epithelial cells, of two lamellas (arrow) (scalebar=10µm, H&E). E) Hypertrophy of lamellar epithelial cells, note the loss of lamellar structure in both lamellas (arrow) and the focal oedemas (arrow head) (scalebar=10µm, TC).

### 4.4.3 Digestive tract

In the digestive tract were only found alterations from the first reaction pattern, including oedemas and infiltration of defence cells, like leukocytes. The overall condition of the digestive tracts was

made through the analysis of the histological condition of three organs, oesophagus, stomach and the intestine. In table 5, are displayed the histopathological traits surveyed in this analysis and the respective histopathological weight.

Table 5 Histopathological alterations observed in the digestive tract of *D. sargus* and their respective condition weight (w)

| Reaction Pattern | Histopathological alteration | Weight |
|---|---|---|
| 1.Circulatory disturbances/ inflammatory responses | Oedema | 1 |
| | Defence cells infiltration | 1 |

No recognizable histopathological lesions could be found in the oesophagus in either blank (B), control (C) or metal exposed fish. The same happened for the analysed stomach, revelling no alterations apart for an occasional focal oedema found in the connective tissue or muscle, but with few histological relevance. In the surveyed intestines, the only histopathological alterations found were an occasional oedema and infiltration of defence cells in the epithelial cells of the mucosa, or in the connective tissue of the submucosa (Fig 14). This trait did not exhibit a trend regardless of treatment type or concentration of metal.

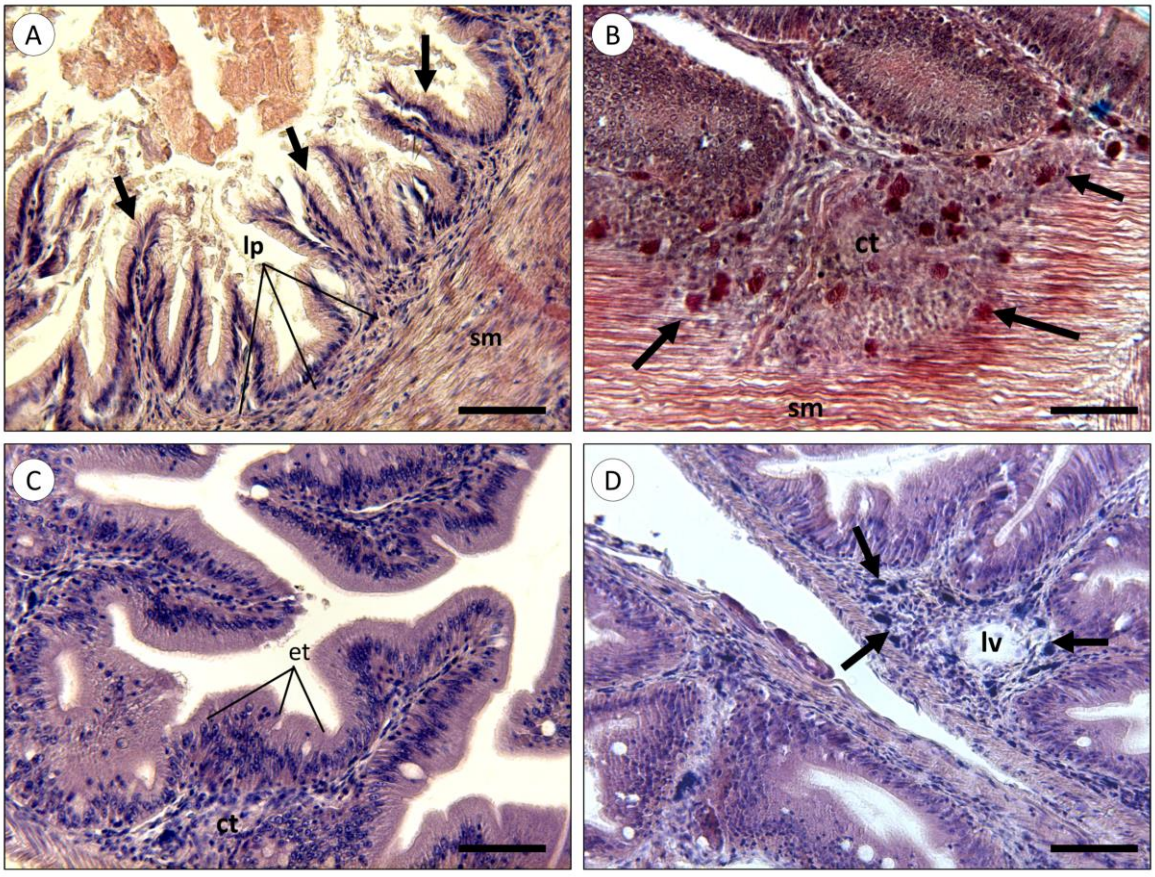



Figure 14 Histopathology of the digestive tract of *D. sargus*: A) Stomach normal condition with the connective tissue (ct) of lamina propria (lp) underlying villi (arrows) and striated mucles (sm) (scalebar=50µm, H&E)). B) Infiltration of defence cells in the stomach, with proliferation of cells (arrows) and consequent swelling of the connective tissue (scalebar=50µm, TC). C) Intestine villi blank condition with a layer of connective tissue (ct), cover by an epithelium made of enterocytes ▶

◀ (et), goblet cells (gb) and microvilli in the apical surface of the epithelial cell (scalebar=50µm, H&E). D) Infiltration of defence cells (arrows) around a lymphatic vessel (lv) (scalebar=50µm, H&E).

### 4.4.4. Spleen

In spleen, only two types of alteration were surveyed, as displayed in Table 6. Spleen normal histological structure was characterized by two different tissues composing the organ, without clear boundaries between them, the red pulp composed primarily of reticulocytes and erythrocytes and the white pulp constituted mostly by lymphoid cells, (Fig.15).

Table 6 Histopathological alterations observed in the spleen of *D. sargus* and their respective condition weight (w)

| Reaction Pattern | Histopathological alteration | Weight |
|---|---|---|
| 1.Circulatory disturbances/Inflammatory responses | Melanomacrophages | 1 |
| 2 Regressive alterations | Necrosis | 3 |

Centres of dark brown (pigmented with melanin) macrophages, melanomacrophage (MMC) (Fig.15B), were frequently observed in all treatments, even in control fish. Those were variable in size and number and did not follow a clear trend regardless of the provided diet. Foci of necrotic cells were found within this organ, but they presented a reduced extension.

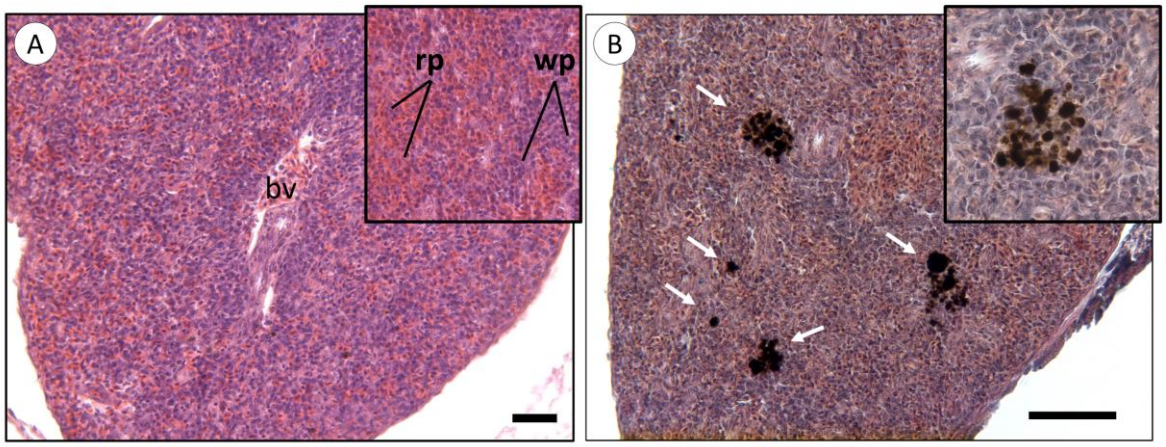



Figure 15 Histopathology of the spleen of *D. sargus*: A Spleen blank condition of the surveyed fish (scale bar= 200µm, H&E) Inset: detail of splenic tissue, with red pulp (rp) and white pulp (wp). B) Splenic tissue with high densities of melanomacrophage *(MMC)* centres (arrows) (scale bar=50 µm, TC). Inset: Detail of MMC aggregate revealed by the dark brown inclusions.

### 4.4.5 Overall condition- Multi-organ index

The overall condition of the individuals was assessed by the construction of an individual global index ($I_{h)}$ composed by all histopathological traits found in the different organs. As theoretically expected, the fish group with minimum medium level of histopathological traits was the blank *mean $I_h$ (B)= 0.136*. In this group was found the minimum $I_h$ of all individuals (0.087) but also the second highest individual value (0.201). The highest mean level of histopathological condition relatively to blank group was observed in the group of the fish exposed to a treatment diet of 0.2 % microplastics coated with antifoulant paints. This group presented a *mean* $I_h$ value (0.177) 23.3% higher than

blanks (B). Among this group was found the maximum value of $I_h$ (0.241). The biggest difference between the medium global indices of fish exposed to metals and their respective control was found in the concentration 0.1%. The *mean* $I_h$ of fish exposed to metal was 17 % higher than the ones exposed only to microplastics ($I_h$(C)=0.142; $I_h$(M)=0.144). To the concentration of 0.2%, the *mean* $I_h$ of fish exposed to metal was 16.3% higher relatively to the respective control fish. ($I_h$ (C)=0.148; $I_h$(M)= 0.177).

### 4.5. Statistical analysis

Statistical analysis revealed no significant differences between the histopathological conditions in the different groups (Kruskal-Wallis H, p>0.05), indicating a similar histopathological condition among the individuals of the different experimental diets. No significant differences could be found (Mann-Whitney U, p>0.05) between the histopathological condition indices of metal exposed fish and the control ones, both for the overall condition of the individuals ($I_h$) and for the indices constructed per organ, this meaning that the apparent trend described before for the $MeanI_h$ being higher in the metal exposed fish than in their respective controls to both concentrations C1 and C2, had an associated error that turned the results inconclusive.

The correlations between the individual histopathological condition and biometric data returned a significant and positive correlation, (R=0.370, p < 0.05) between the overall histopathological condition and the weight, the same positive correlation was found with the fish length (R= 0.399 p<0.05) and its overall histopathological condition. The correlation between the histopathological condition of each organ, with the biometric data, only generated significant correlation for the liver, corresponding to a positive correlation (R= 0.460, p<0.05 to the weight, and R=0.454 p<0.05 for the length).

The comparison between the degree of affectation in the different surveyed organs revealed the significant differences between all organs (p<0.05). Hepatopancreas recorded the highest degree of affectation, mainly because of hepatic parenchyma being the most altered tissue in the organisms (Fig. 16A) Gills were the second most affected organ, followed by the spleen. The organs constituting the digestive tract and the pancreatic tissue were the least affected organs. Overall the comparison between the organs returned a degree of affectation corresponding to Hepatic tissue > Gills> Spleen > Pancreatic tissue> Gut. This relation was not exactly the same in all groups, in some of them (C1 with metals and the controls of C2), the gut appeared to be more affected than spleen or pancreatic tissue but the most affected organs (liver and gills) were the same in all groups.

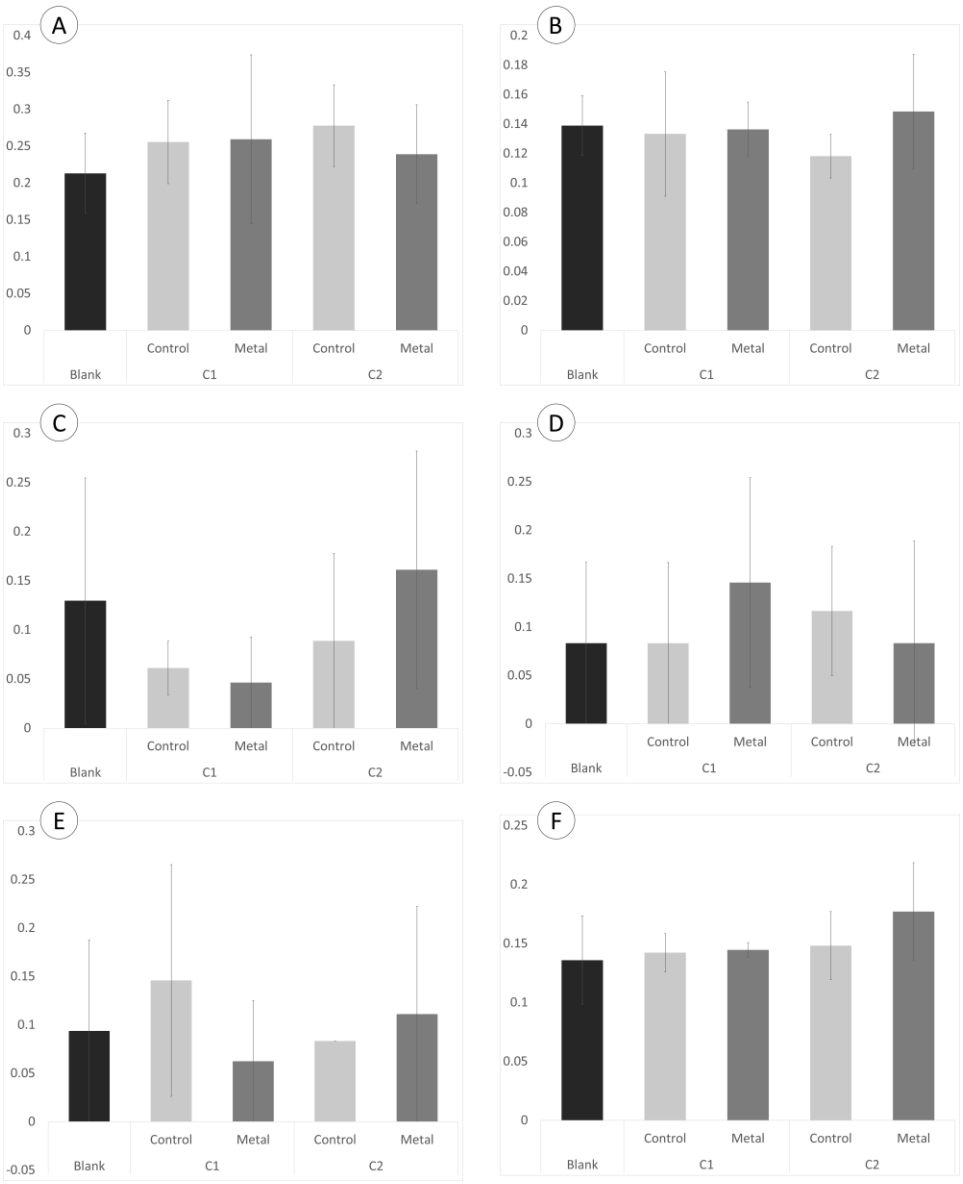



Figure 16 Histopathological condition indices in the different groups (error bar= sd): A) Hepatic Index. B) Gills Index. C) Pancreatic index. D) Gut index. E) Spleen index. F) Global histopathological condition index. No significant differences were found between groups in any the of the constructed indices.

# 5. Discussion

The present findings indicate null or inconclusive effects of microplastics as vector for metal contamination. The results show that the microplastic pellets did not seem to increase the bioavailability of metal that could result in significant adverse effects in the organisms, including putative metal deposits. The fish clearly ingested the microplastic pellets, evidenced by the particles present in both stomach and intestine (recall Fig. 1). Still, no negative and significative effects were found in these organs, including inflammatory response-related alterations. This suggests that *Diplodus sargus* are capable of ingesting and eliminate smooth microplastic pellets within a diameter size of 700-900 µm, without major deleterious consequences. Contrarily to the fish, *Palaemon serratus* did not seems to ingest this type of particles and no significant histopathological alterations could be pin-pointed to exposure. In spite of the differences in response to the bioassay, in both species, the comparison between organisms exposed to coated microplastics and their respective controls, revealed no significant differences in the occurrence of histopathological traits Microplastic pellets are not just capable to adsorb this type of metals from antifouling paints, as reported in Brennecke et al. (2016), but also they have the capacity to increase metals bioaccessibility to marine organisms as reported in Holmes (2013). Contaminated pellets yield two routes of metal exposure, dietborne due to metal release from the pellets upon ingestion, and waterborne due water contamination thru leached metals from the pellets into the saltwater. However, no significative negative effects were found within the surveyed organisms. Therefore, it is possible that the bioavailability of the metals both in water and organisms has remained unaltered, due to external factors or at the same time, it is possible that the existing increase of bioavailability of these metals may have been completely regulated by the organisms without major consequences.

The toxicity of both copper and zinc to marine organisms, from exposures through metal-contaminated water, is influenced by two key factors: 1) the metal leach rates from the paint, since they regulate the quantity of metals released into the water and 2) metal speciation, known to be determinant in both copper and zinc bioavailability. Regarding the leach rates, the used paint is a two-part antifouling paint system, constituted by Micron Optima Base YBA953 with a copper leach ranging between 9.5 and 13.4 µg/cm2/day (Gutierrez, 2017) and Micron Activator YBA953 with a copper leach rate below or equal to (≤) 9.5 µg/cm2/day (Gutierrez, 2017), these rates were calculated accordingly to the (ISO) 10890:2010. Accordingly, to these values, was estimated a daily input of copper into the water within 0.15 and 1.2 µg Cu. Still it must be highlighted that in this study the metals leach rates were not evaluated, and that the used standard leach rate might have been influenced by several factors such as the water flow, temperature, pH, salinity (Brooks, 2009) and the presence of microbial populations (Meseguer Yebra et al. 2006). In regard to speciation of copper and zinc, apart from the physical and chemical characteristics, such as pH, alkalinity, ion concentrations known to influence the bioavailability of metals in general (Brooks, 2009), the presence of DOC and high concentrations of phosphate, seem to have a major influence in decreasing copper and zinc bioavailability to organisms (Clearwater et al. 2002) Considering the exposure apparatus, specifically the filter system it is not expected that there was a high concentration of DOC or even phosphate during the bioassay, which could have reduced significantly the bioavailability of metals.

Holmes (2013) reported a rapid desorption of metals adsorbed to plastic ingested pellets, within a simulated digestive tract of a marine avian, demonstrating a high bioaccessability, up to 83% for zinc, of all analysed metals. However, there is a lack of knowledge about the factors that may influence the toxicity of this metals within the digestive tract. The factors that affect bioavailability probably differ from waterborne exposures, but they might be the same as the ones found in studies carrying another type of diets (vectors) to dietborne origin of contamination, such as formulated diets spiked with Cu and Zn salts (Spry et al. 1998) or natural diets composed of contaminated organisms (Mount et al. 1994; Sofyan et al. 2006). Apart from the known decrease in Zn bioavailability, in organisms feed with high doses of phosphates and phytates (Davis, 1996), there is lack of information about other external factors that may influence metals availability upon ingestion. It is suggested that artificially contaminated diets (as the one provided) may decrease metals availability, in contrast to natural contaminated diets (Grosel, 2011). Overall metal uptake and metabolization is highly differentiated not only among related species but also between different tissues of the same species. (Rainbow, 1997) therefore is difficult to identify and quantify the interference of external factor in metal exposure.

In addition to factors that may have influenced metal availability, both copper and zinc are essential oligonutrients for fish and shrimps. The toxicity thresholds of these metals are, therefore, presumably high, which may explain, at least in part, reduced adverse effects in either species. Copper and Zn requirements differ among species and within the life stages of the same species. In both metals, the requirements seem to be higher in shrimps, than in fish (Davis, 1996; Ali et al. 2006). Therefore, shrimps have probably higher tolerance to both dietborne and waterborne exposures of this metals, this may explain why shrimps presented very few histopathological traits when compared to fish. Altogether the literature suggests an ability of decapod crustaceans to regulate internal concentrations of both metals when exposed to moderated-high concentrations. For example, *P. elegans*, is known to regulate its internal concentration of Cu and Zn holding them constant when exposed to concentration up to 100 µg/l (White and Rainbow, 1982). The same ability to keep internal metal homeostasis, without associated deleterious effects, has been reported in many fish, from the marine *Sparus aurata* (Serra et al. 1996; Minghetti et al. 2008), a fish from the same family of *D. Sargus* to the freshwater rainbow trout (Spry et al. 1998).

As expected the most affected organ was the fish hepatopancreas, specifically the hepatic parenchyma. The hepatic tissue in most teleost fish has been shown as the most important organ in the metabolism of xenobiotics, including metals. However, even in this organ the relation between histopathological traits and metal contaminated pellets was not evident. In one hand the surveyed biomarkers are completely non-specific not revealing a clear relation to metal exposures and at the same time, the positive and significative correlations found between the histopathological condition of the hepatic tissue and the biometric data of the fish may indicate a significant effect of the aging process in fish and its hepatic condition, as reported previously by Stentiford et al. (2010) and Cuevas et al. (2015). Overall the least affected organs were the ones in the digestive tract. In one hand, this could significate a higher exposure through contaminated water than through the ingestion of coated microplastics and consequently higher effect of other organs. But at the same time, the digestive tract, of teleost fish specially the intestine has a documented role in uptake and metabolism of metals (Clearwater et al. 2002). this meaning this organ is able to lead with metals regularly, and consequently has structures able to protect the intestinal tissues from dietborne

metal exposure, camouflaging the degree of exposure. In fact, dietborne exposures of Cu and Zn seem to be less toxic to aquatic organisms, than waterborne exposures (Schlekat et al. 2001; Clearwater et al. 2002; Deforest and Meyer, 2014). This possibly explains why metal deposits were more common in epithelial cells of the intestine mucosa than in gills. (Fig.2), and yet this organ did not reveal significant traits. Mucus is known to play an important role in the protection of the internal tissues and as a pathway of metal uptake and excretion, (Clearwater et al. 2002; Campbell et al. 2005), explaining why metal deposits were found frequently circumscribed in goblet cells of the intestinal epithelium, as well as in the opercular cavity (Fig.2A/C).

The differences in plastic ingestion might be explained by different feeding behaviours and morphology. *Diplodus sargus*, like other sparidae, are benthic omnivores (Coetzee, 1986, Figueiredo et al. 2005) with unselective and opportunistic behaviour (Gonçalves and Erzini, 1998) that results in the suction of all types of food. The morphological and behavioural characteristics of the feeding mechanisms of this species, like the existence of differentiated ingestion stages and the presence of differentiation dentition (Vandewalle et al. 1995), allows the predation on larger hard-bodied preys (Coetzee, 1986; Figueredo et al. 2005) including shell-fish, indicating an ability of this species to eliminate hard objects without adverse effects. The highlighted traits can explain why fish ingested the microplastic pellets, and why this ingestion did not produce any negative effects on the digestive tract, not even in organisms where 2-3 pellets were found in the same intestine section (recall Fig.1.B). The smooth spherical shape of commercial pellets (i.e. devoid of edges) seems to help the elimination without interferences in the digestive and the pellet size (700-900µm), do not seem to be a stress. On the other hand, the feeding behaviour of *Palaemon* seems to be very influenced by the local availability of food and the size of the individuals (Guerao and Ribera, 1996). In juveniles, *Palaemon* spp. are omnivorous scavengers, with no selective food capture but with a selective process of ingestion. The feeding appendix of this species have a major role in this selectivity trough food manipulation and examination (Barros and Valenti, 1997), allowing acceptance or rejection of food before entering the mouth (Felgenhauer and Abele, 1983). In addition, the internal digestive tract of this species is prepared to expel regularly detritus incapable to digest, since only liquid form of digesta reaches the midgut (Mcgaw, 2013). Forster and Gabbott, (1971) described, regurgitating part of their gut contents following each meal as a habit of the *P. serratus* species in laboratory conditions. These traits might explain why no plastic pellets could be found in any of the surveyed shrimps. In one hand, the plastic may have been rejected through food manipulation and selectivity and at the same time, if ingested the plastic pellets could have been all regurgitated since they were impossible to digest.

It must be highlighted that the conditions of the bioassays, especially of mid-long duration, as in the case, may result in significant confounding factors. The fact that all animals were collected from nature, may bring in a higher variability of background diseases impossible to track. For example, most fish showed clear sign of myxozoa infection, giving rise to PKD. Considering the severity of this disease (Alvarez-Pellitero, 2004), it is probable that the histological condition of the entire organism has been compromised, at least in part, which might have performed as a confounding factor in the semi-quantitative analysis, by increasing the occurrence of histopathological traits in other organs, which helps to explain the high values of $I_h$ found in the blanks. At the same time the exclusion of a very important organ in the internal regulation of metals via filtration and excretion, besides key role in the piscine immune system, probably affected the accuracy of the analysis.

# 6. Conclusions

The current findings indicate that under the circumstances of this study, which aim to represent ecologically relevant scenarios of contamination (without unrealistic doses of microplastics or metal inputs) the vector effect of microplastics was not significative since it was impossible to associate adverse effect, or metal depositions to the exposition to metal contaminated pellets.

These coastal and juvenile organisms seem to be little affected by this type of pellets. The shrimp did not ingested plastic, probably due to a particular selectivity during the ingestion process resultant from its morphoanatomy of the digestive tract and its feeding behaviour that enable the rejection of this type of microplastic pellets without compromising the ingestion of the food content attached to the pellets. The fish clearly have the capacity to ingest particles with this size, however, they seem to be anatomically equipped to eliminate solid food particles, at least this type of smooth spheres (devoid of edges). What may happen with naturally fragmented microplastics, likely exhibiting rough edges, is unknown.

Under the specific circumstances of this study, using microplastic pellets coated with antifouling paints, the release of Cu and Zn from the plastic matrix is not critical. However, it must be highlighted, that using a paint as a proxy of metal contamination in plastic is not the same of using microplastics with adsorbed metals, as it naturally occurs.

These results highlight the importance of carrying out more studies to understand the processes of desorption of metals from plastic materials, coated or not within the water column and specially within digestive tract of marine organisms, where the effect of acidity, enzymes and tissues speciality may severely influence the bioavailability, which may depend not only on the plastic matrix but also from the type of metal and the associated speciation processes.

In future perspectives, more prolonged exposures with organism within different stages of life may complement the results of this study, and confirm or not the low susceptibility of this species to coated microplastic pellets. Furthermore, tests with more realistic materials, such as plastic microfragments instead of virgin materials and/or naturally contaminated materials may provide a more accurate description from what really happens in marine environment. In addition, future studies should focus in controlling, some confounding factors, at least in part, the use of wild organisms, brings in more intraspecific variability and more variability in background diseases. Finally, the analyses of other type of biomarkers, such as behavioural and biochemicals indicators may be helpful for access effects on the organism performance and health during the bioassay, thus contributing to more accurate results.

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